Synthesis of a Low-Molecular-Weight Form of Exopolysaccharide by *Bradyrhizobium japonicum* USDA 110

HEATHER A. LOUCH¹† AND KAREN J. MILLER^{1,2}*

Intercollege Graduate Program in Genetics¹ and Department of Food Science,² The Pennsylvania State University, University Park, Pennsylvania 16802

Received 3 July 2000/Accepted 4 December 2000

A novel extracellular low-molecular-weight polysaccharide was detected as a contaminant within extracellular cyclic β -1,6- β -1,3-glucan preparations from *Bradyrhizobium japonicum* USDA 110 cultures. Compositional analysis, methylation analysis, and nuclear magnetic resonance analysis revealed that this low-molecular-weight polysaccharide was composed of the same pentasaccharide repeating unit previously described for the high-molecular-weight form of the exopolysaccharide (EPS) synthesized by *B. japonicum* strains. Mass spectrometry analysis indicated that the size of this low-molecular-weight form of EPS was consistent with a dimeric form of the pentasaccharide repeating unit.

Gram-negative soil bacteria of the family *Rhizobiaceae* synthesize a variety of cell-surface carbohydrates. These carbohydrates include lipopolysaccharides, capsular polysaccharides, exopolysaccharides (EPS), nodule polysaccharides, lipo chitin oligosaccharides, and cyclic β -glucans, some of which may provide functions important to symbiosis (1, 8, 13, 19, 21). These cell-surface carbohydrates may also have important functions for the free-living forms of these bacteria. For example, both cyclic β -glucans and EPS are believed to play roles during osmotic adaptation, and levels of these carbohydrates have been shown to be directly influenced by the osmotic strength of the growth environment (3, 4, 5, 8, 14, 21, 22).

During the past several years, research in our laboratory has focused on the cyclic β -glucans of bacteria within the *Sinorhizobium* and *Bradyrhizobium* genera (8, 9). In *Sinorhizobium* species, the cyclic β -glucans are linked solely by β -1,2 glycosidic bonds, whereas in *Bradyrhizobium* species, these molecules are linked by both β -1,3 and β -1,6 glycosidic bonds. The cyclic β -glucans are localized within the periplasmic compartment of these bacteria, but they are also released into the medium. Indeed, we have previously shown that *Bradyrhizobium japonicum* cultures excrete relatively high levels of cyclic β -1,6- β -1,3-glucans into the culture medium, with levels approaching up to sevenfold higher than levels associated with cells (22). As part of our ongoing research efforts, we recently developed a radiolabel screening strategy to isolate mutants of *B. japonicum* impaired for the synthesis of cyclic β -1,6- β -1,3-glucan (H. A. Louch and K. J. Miller, Abstr. 95th Gen. Meet. Am. Soc. Microbiol. 1995, abstr. N-197, p. 366, 1995). During the course of screening putative cyclic β -glucan mutants of *B. japonicum* USDA 110, a novel, low-molecular-weight form of EPS was identified which copurified with extracellular cyclic β -1,6- β -1,3-glucans. The purification and structural analysis of

this low-molecular-weight form of EPS are described in the present study.

Identification of *B. japonicum* mutant M1E7. Tn5 mutagenesis of *B. japonicum* USDA 110 was performed using a biparental mating procedure, essentially as described by Hom and coworkers (17). Tn5 mutants were plated onto GMS (32) containing (per ml) 100 μ g of streptomycin, 100 μ g of kanamycin, and 50 μ g of trimethoprim. Approximately 2,100 Tn5 mutants of *B. japonicum* USDA 110 were isolated, and each was screened for defects in cyclic β -1,6- β -1,3-glucan biosynthesis as described below.

Each *B. japonicum* USDA 110 Tn5 mutant was inoculated into 5 ml of GMS medium. Cultures were grown to an optical density at 650 nm of between 0.3 and 0.6 in a 30°C rotary shaker (7 to 8 days on average), at which time radiolabeled glucose (either [6 - 3 H]glucose or [14 C]glucose) was added to a final concentration of 100 μ M and at a specific activity of 0.5 μ Ci/ml. Cultures were incubated for 3 to 6 h in the presence of radiolabel. After incubation, cells were pelleted by centrifugation (12,000 $\times$ g for 5 min) and washed twice with water (1 ml), and cyclic β -1,6- β -1,3-glucans were extracted with 160 μ l of 70% ethanol at 70°C for 30 min. The level of radiolabeled cyclic β -1,6- β -1,3-glucans within each ethanol extract was determined by adsorption onto C₁₈ silica gel resin (Supelco, Bellefonte, Pa.) followed by selective elution using 30% methanol. This screening strategy using C₁₈ silica gel resin was based on an earlier report by Rolin and coworkers (28) that revealed that cyclic β -1,6- β -1,3-glucans could be bound to C₁₈ silica gel resin and selectively eluted using 30% methanol. Of approximately 2,100 mutants screened, 1 mutant, referred to as mutant M1E7, was found to contain extremely low levels of radiolabel in the 30% methanol eluent (i.e., 4% of the level produced by the parent strain, USDA 110). Based on this finding, mutant M1E7 was selected for further study.

Analysis of extracellular low-molecular-weight polysaccharides from *B. japonicum* cultures. *B. japonicum* strains were cultured in 500 ml of YM medium (23) at 30°C until reaching an optical density at 650 nm of 0.6. Cells were harvested (13,000 $\times$ g for 10 min) and washed with 25 ml of YM salts buffer at pH 7.0 (23), and culture supernatants were frozen.

* Corresponding author. Mailing address: 105 Borland Laboratory, Department of Food Science, The Pennsylvania State University, University Park, PA 16802. Phone: (814) 863-2954. Fax: (814) 863-6132. E-mail: kjm3@psu.edu.

† Present address: Laboratory of Bacterial Toxins, Center for Biologics Evaluation and Research, Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20892.

FIG. 1. Negative-ion FABMS analysis of the extracellular low-molecular-weight, anionic polysaccharide isolated from *B. japonicum* mutant M1E7 culture supernatants. The major molecular ion species had m/z values of 1,791, 1,806, 1,833, 1,848, and 1,862. Negative-ion FABMS analysis was performed using a VG Analytical ZAB-2SE high-field mass spectrometer (Micromass Ltd., Manchester, United Kingdom). A cesium gun was used to bombard the sample, which was analyzed in a matrix of glycerol-thioglycerol-triethylamine. Analysis of samples was performed at M-Scan Inc. (West Chester, Pa.).

After thawing, culture supernatants were concentrated 25-fold by rotary evaporation. Next, high-molecular-weight EPS was precipitated from concentrated supernatants by adding 3 volumes of ice-cold ethanol as described by Breedveld and co-workers (10). High-molecular-weight EPS was then removed from concentrated supernatants by centrifugation ($12,000 \times g$ for 10 min). Low-molecular-weight, ethanol-soluble polysaccharides were then purified from concentrated supernatants using gel permeation chromatography as described below.

Concentrated supernatants containing ethanol-soluble, extracellular low-molecular-weight polysaccharides were concentrated under vacuum. Samples were applied to a Sephadex G-25 column (1 by 52 cm) which was eluted at room temperature with 0.15 M ammonium acetate (pH 7.0) containing 7% propanol (vol/vol) at a rate of 15 ml/h. Fractions (1 ml) were collected and assayed for carbohydrate content (12). Material eluting in the position expected for cyclic β -1,6- β -1,3-glucan was pooled, concentrated, and subsequently desalted using a Sephadex G-15 column (1 by 49 cm). The Sephadex G-15 column was eluted at room temperature with 7% propanol (vol/vol) at a rate of 15 ml/h. Fractions (1 ml) were collected and assayed for carbohydrate content. Material eluting in the position expected for cyclic β -1,6- β -1,3-glucan was pooled and subsequently analyzed by thin-layer chromatography (TLC) using aluminum-backed Silica Gel 60 plates (EM Industries, Gibbstown, N.J.) and a butanol-ethanol-water (5:5:4) solvent system. Samples were visualized on TLC plates by charring at 170°C for 20 min after spraying with 5% sulfuric acid in methanol (vol/vol) (7).

Identification of a novel, low-molecular-weight form of EPS within *B. japonicum* cultures. TLC is routinely performed in our laboratory to monitor the purification of cell-associated

cyclic β -1,6- β -1,3-glucans from *B. japonicum* cultures; however, it can also be used to monitor the purity of cyclic β -1,6- β -1,3-glucans obtained from the supernatants of *B. japonicum* cultures. When the low-molecular-weight, extracellular polysaccharides of *B. japonicum* mutant M1E7 were examined by TLC, a major spot, migrating with a substantially higher R_f value than the cyclic β -1,6- β -1,3-glucans, was detected (data not shown). Indeed, this material was a major contaminant within cyclic β -1,6- β -1,3-glucan preparations and represented approximately 75% (glucose equivalent) of the total carbohydrate present within the low-molecular-weight fraction isolated from culture supernatants of mutant M1E7. Further analysis revealed that this contaminant could be bound to DEAE-cellulose at pH 8.4 and subsequently eluted using a buffer containing 200 mM KCl, indicative of anionic character. This is in contrast to the cyclic β -1,6- β -1,3-glucans, which are uncharged and do not bind to DEAE-cellulose under these conditions.

Additional characterization of the extracellular anionic contaminant material isolated from mutant M1E7 was performed using negative-ion fast atom bombardment mass spectrometry (FABMS). FABMS analysis revealed a mass spectrum distinctly different from that obtained for the cyclic β -1,6- β -1,3-glucans. For example, it has previously been shown that a typical mass spectrum for purified cyclic β -1,6- β -1,3-glucan contains predominant molecular ion species ($[M-H]^-$) at m/z values of 1,619, 1,781, 1,943, and 2,005 (23), which correspond to unsubstituted cyclic glucans containing 10 to 13 glucose residues. However, when the anionic, low-molecular-weight extracellular polysaccharide material obtained from mutant M1E7 was examined by negative-ion FABMS, the analysis revealed a very different spectrum, although the predominant molecular ion species had m/z values in the same range as the

TABLE 1. Compositional analysis of the extracellular, low-molecular-weight, anionic polysaccharide from *B. japonicum* M1E7 and USDA 110^a

<i>B. japonicum</i> strain	Concn (M), normalized to mannose			
	Mannose	Glucose	Galacturonic acid	Galactose
M1E7	1.00	2.00	0.81	0.81
USDA 110	1.00	2.01	1.09	0.51

^a The extracellular anionic low-molecular-weight polysaccharide was separated from cyclic β -1,3- β -1,6-glucans using TLC. Low-molecular-weight EPS was eluted from the TLC plate with water and then desalted on a Sephadex G-15 column. Compositional analysis was performed using gas chromatography linked to electron impact mass spectrometry after hydrolysis, *N*-acetylation, and derivatization with trimethylsilyl (TMS). The composition is the same as that previously reported for the high-molecular-weight EPS of *B. japonicum* strains (18, 24, 25, 27). Samples and standards (fucose, xylose, mannose, galactose, glucose, *N*-acetylgalactosamine, *N*-acetylglucosamine, *N*-acetylneuraminic acid, rhamnose, and galacturonic acid) were hydrolyzed using methanolysis. Potential amino sugars were then re-*N*-acetylated, and the products were finally derivatized with TMS to render them volatile for gas chromatography-mass spectrometry analysis. The analysis was performed using a VG TRIO 1 instrument (Micromass Ltd.). Arabitol was used as an internal standard. Analyses were performed at M-Scan Inc.

cyclic β -1,6- β -1,3-glucans (Fig. 1). This result confirmed that the anionic low-molecular-weight polysaccharide material is very similar in size to the cyclic β -1,6- β -1,3-glucans, consistent with the fact that these materials copurify on Sephadex G-25 and Sephadex G-15.

A compositional analysis of the extracellular low-molecular-weight polysaccharide isolated from the *B. japonicum* M1E7 mutant was performed using gas chromatography linked to electron impact mass spectrometry. As shown in Table 1, the material was found to contain glucose, mannose, galacturonic acid, and galactose, in a 2:1:1:1 ratio. This is the same composition previously reported for the high-molecular-weight EPS of *B. japonicum* strains (18, 24, 25, 27). Based on these results, additional analyses were performed on the low-molecular-weight anionic polysaccharide material obtained from culture supernatants of the wild-type parent strain, USDA 110. These results are also shown in Table 1 and reveal that both mutant M1E7 and the wild-type parent strain, USDA 110, produce and excrete a low-molecular-weight form of EPS.

Further structural examination of the putative low-molecular-weight EPS was performed at the Complex Carbohydrate Research Center at the University of Georgia (Athens) using methylation and gas chromatography-mass spectrometry analysis (as described by Ciucanu and Kerek [11]), as well as 1-D ¹H nuclear magnetic resonance (NMR) analysis. Methylation analysis revealed the presence of terminally linked galactose, 3-linked glucose, 3-linked mannose, and 3,6-linked glucose. Unfortunately, methylation analysis could not be used to identify uronic acid residues; thus, galacturonic acid could not be detected. However, NMR analysis revealed the presence of an *O*-methyl group, an *O*-acetyl group, and resonances consistent with a 4-*O*-acetylated galacturonic acid residue (Fig. 2). Thus, the combined results of the methylation and NMR analyses are fully consistent with the structure of the pentasaccharide repeating unit previously reported for *B. japonicum* EPS (24, 25). The NMR spectra previously published for high-molecular-weight EPS of *B. japonicum* strains is in good agreement with our spectrum (25, 27). Based on the apparent size of this low-molecular-weight polysaccharide, as indicated by gel per-

FIG. 2. 1-D ¹H NMR spectrum of the extracellular, low-molecular-weight, anionic polysaccharide from *B. japonicum* USDA 110 culture supernatants. The resonances are identified as follows: 1 = H4 of the alpha-linked 4-*O*-methyl galacturonic acid; 2 = H1 of the alpha-linked 4-*O*-acetyl galacturonic acid and H1 of the alpha-linked glucose residues; 3 = H1 of the alpha-linked mannose residue; 4 = H1 of the alpha-linked 4-*O*-methyl galactose residue; 5 = methyl protons of a 4-*O*-methyl group; and 6 = methyl protons of an *O*-acetyl group. The H1 residue of the beta-glucose is obscured by the HOD (water) peak at 4.7 to 4.8 ppm. The remaining resonances between 3.5 and 4.7 ppm that are not labeled are due to other ring protons of the various glycosyl residues. The resonances labeled with asterisks were not identified but are not due to carbohydrates. Analyses were performed using a Varian 300 MHz instrument at the Complex Carbohydrate Research Center at the University of Georgia (Athens).

meation chromatography and negative-ion FAB/MS, it may be concluded that this material corresponds to a dimeric form of the pentasaccharide repeating unit of the *B. japonicum* EPS.

Concluding remarks. To our knowledge, this is the first study to demonstrate the presence of a low-molecular-weight form of EPS in *B. japonicum* cultures, although it should be noted that Becker and coworkers (6) previously provided preliminary evidence for a low-molecular-weight form of EPS in *B. japonicum* 110spc4 cultures. The finding of a low-molecular-weight form of EPS in *B. japonicum* cultures was not surprising, since low-molecular-weight forms of EPS have previously been identified in *Sinorhizobium meliloti* cultures (2, 14). In fact, the low-molecular-weight forms of EPS produced by *S. meliloti* have been shown to promote nodulation of alfalfa by this symbiont (2, 14). In future studies, it will be of interest to examine whether or not the low-molecular-weight form of the *B. japonicum* EPS influences the nodulation of the soybean host, particularly since studies have indicated that effective symbioses are possible with mutants that do not synthesize the high-molecular-weight form of EPS (20, 26).

Our discovery of a low-molecular-weight form of *B. japonicum* EPS was initially made with *B. japonicum* mutant M1E7, a Tn5 mutant of USDA 110. This mutant was selected for further study because radiolabeling studies suggested that it was impaired for cyclic β -1,6- β -1,3-glucan biosynthesis. Curiously, analyses of nonradiolabeled, large-scale cultures of M1E7 revealed that the levels of cell-associated cyclic β -1,6- β -1,3-glucans are similar to the levels found in wild-type USDA 110 cultures (data not shown). However, these analyses

have also revealed that mutant M1E7 produces approximately twofold higher levels of extracellular, low-molecular-weight polysaccharides when compared to the wild-type USDA 110 strain. Therefore, it is possible that the low level of radiolabeled glucose incorporated into the cyclic β -1,6- β -1,3-glucan pool of M1E7 during the radiolabel screening procedure was a result of increased extracellular polysaccharide production by this mutant.

Further analysis of mutant M1E7 revealed another curious result. Specifically, the Tn5 insertion was found to lie within the *clpA* gene (the *B. japonicum* USDA 110 *clpA* sequence as well as the location of Tn5 within the *clpA* gene of mutant M1E7 are described within GenBank accession no. AF254897). ClpA is the regulatory subunit of the ClpAP protease, an ATP-dependent protease involved in the turnover of abnormal proteins (16, 29, 31). The results of the present study suggest a possible role for the ClpAP protease in the regulation of bacterial polysaccharide production. In this regard, it is interesting to note that the Lon protease has previously been shown to have a regulatory role in capsular polysaccharide biosynthesis in *Escherichia coli* (15, 30).

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REFERENCES

- An, J., R. W. Carlson, J. Glushka, and J. G. Streeter. 1995. The structure of a novel polysaccharide produced by *Bradyrhizobium japonicum* species within soybean nodules. *Carbohydr. Res.* **269**:303–317.
- Battisti, L., J. C. Lara, and J. A. Leigh. 1992. Specific oligosaccharide form of the *Rhizobium meliloti* exopolysaccharide promotes nodule invasion in alfalfa. *Proc. Natl. Acad. Sci. USA* **89**:5625–5629.
- Becker, A., H. Küster, K. Niehaus, and A. Pühler. 1995. Extension of the *Rhizobium meliloti* succinoglycan biosynthesis gene cluster: identification of the *exsA* gene encoding an ABC transporter protein, and the *exsB* gene which probably codes for a regulator of succinoglycan biosynthesis. *Mol. Gen. Genet.* **249**:487–497.
- Becker, A., and A. Pühler. 1998. Production of exopolysaccharides, p. 97–118. In H. P. Spink, A. Kondorosi, and P. J. J. Hooykaas (ed.), *The Rhizobiaceae: molecular biology of model plant-associated bacteria*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Becker, A., and A. Pühler. 1998. Specific amino acid substitutions in the proline-rich motif of the *Rhizobium meliloti* ExoP protein result in enhanced production of low-molecular-weight succinoglycan at the expense of high-molecular-weight succinoglycan. *J. Bacteriol.* **180**:395–399.
- Becker, B. U., K. Kosch, M. Parniske, and P. Müller. 1998. Exopolysaccharide (EPS) synthesis in *Bradyrhizobium japonicum*: sequence, operon structure and mutational analysis of an *exo* gene cluster. *Mol. Gen. Genet.* **259**:161–171.
- Breedveld, M. W., J. A. Hadley, and K. J. Miller. 1995. A novel cyclic β -1,2-glucan mutant of *Rhizobium meliloti*. *J. Bacteriol.* **177**:6346–6351.
- Breedveld, M. W., and K. J. Miller. 1994. Cyclic β -glucans of members of the family *Rhizobiaceae*. *Microbiol. Rev.* **58**:145–161.
- Breedveld, M. W., and K. J. Miller. 1998. Cell-surface β -glucans, p. 81–96. In H. P. Spink, A. Kondorosi, and P. J. J. Hooykaas (ed.), *The Rhizobiaceae: molecular biology of model plant-associated bacteria*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Breedveld, W. B., C. Dijkema, L. P. T. M. Zevenhuizen, and A. J. B. Zehnder. 1993. Response of intracellular carbohydrates to a NaCl shock in *Rhizobium leguminosarum* biovar *trifolii* TA-1 and *Rhizobium meliloti* SU-47. *J. Gen. Microbiol.* **139**:3157–3163.
- Ciucanu, I., and F. Kerek. 1984. A simple and rapid method for the permethylation of carbohydrates. *Carbohydr. Res.* **131**:209–217.
- Daniels, L., R. S. Hanson, and J. A. Phillips. 1993. Chemical analysis, p. 512–554. In P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), *Methods for general and molecular bacteriology*. American Society for Microbiology, Washington, D.C.
- Eppe, G., K. M. G. M. van der Drift, J. E. Thomas-Oates, and O. Geiger. 1998. Characterization of a novel acyl carrier protein, RkpF, encoded by an operon involved in capsular polysaccharide biosynthesis in *Sinorhizobium meliloti*. *J. Bacteriol.* **180**:4950–4954.
- González, J. E., B. L. Reuhs, and G. C. Walker. 1996. Low molecular weight EPS II of *Rhizobium meliloti* allows nodule invasion in *Medicago sativa*. *Proc. Natl. Acad. Sci. USA* **93**:8636–8641.
- Gottesman, S., and V. Stout. 1991. Regulation of capsular polysaccharide synthesis in *Escherichia coli* K12. *Mol. Microbiol.* **5**:1599–1606.
- Gottesman, S., C. Squires, E. Pichersky, M. Carrington, M. Hobbs, J. S. Mattick, B. Dalrymple, H. Kuramitsu, T. Shiroza, T. Foster, W. P. Clark, B. Ross, C. L. Squires, and M. R. Maurizi. 1990. Conservation of the regulatory subunit for the Clp ATP-dependent protease in prokaryotes and eukaryotes. *Proc. Natl. Acad. Sci. USA* **87**:3513–3517.
- Hom, S. S., S. L. Uratsu, and F. Hoang. 1984. Transposon Tn5-induced mutagenesis of *Rhizobium japonicum* yielding a wide variety of mutants. *J. Bacteriol.* **159**:335–340.
- Huber, T. A., A. K. Agarwal, and D. L. Keister. 1984. Extracellular polysaccharide composition, ex planta nitrogenase activity, and DNA homology in *Rhizobium japonicum*. *J. Bacteriol.* **158**:1168–1171.
- Kannenberg, E. L., B. L. Reuhs, L. S. Forsberg, and R. W. Carlson. 1998. Lipopolysaccharides and K-antigens: their structures, biosynthesis, and functions, p. 119–154. In H. P. Spink, A. Kondorosi, and P. J. J. Hooykaas (ed.), *The Rhizobiaceae: molecular biology of model plant-associated bacteria*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Kosch, K., A. Jacobi, M. Parniske, D. Werner, and P. Müller. 1994. The impairment of the nodulation process, induced by a *Bradyrhizobium japonicum* exopolysaccharide mutant is determined by the genotype of the host plant. *Z. Naturforsch. Sect. C* **49**:727–736.
- Leigh, J. A., and D. L. Coplin. 1992. Exopolysaccharides in plant-bacterial interactions. *Annu. Rev. Microbiol.* **46**:307–346.
- Miller, K. J., and R. S. Gore. 1992. Cyclic β -(1,6)(1,3)-glucans of *Bradyrhizobium japonicum*: functional analogs of the cyclic β -(1,2)-glucans of *Rhizobium*? *Curr. Microbiol.* **24**:101–104.
- Miller, K. J., R. S. Gore, R. Johnson, A. J. Benesi, and V. N. Reinhold. 1990. Cell-associated oligosaccharides of *Bradyrhizobium* spp. *J. Bacteriol.* **172**:136–142.
- Mort, A. J., and W. D. Bauer. 1980. Composition of the capsular and extracellular polysaccharides of *Rhizobium japonicum*: changes with culture age and correlations with binding of soybean seed lectin to the bacteria. *Plant Physiol.* **66**:158–163.
- Mort, A. J., and W. D. Bauer. 1982. Application of two new methods for cleavage of polysaccharides into specific oligosaccharide fragments: structure of the capsular and extracellular polysaccharides of *Rhizobium japonicum* that bind soybean lectin. *J. Biol. Chem.* **257**:1870–1875.
- Parniske, M., P. E. Schmidt, K. Kosch, and P. Müller. 1994. Plant defense responses of host plants with determinate nodules induced by EPS-defective *exoB* mutants of *Bradyrhizobium japonicum*. *Mol. Plant-Microbe Interact.* **7**:631–638.
- Puvanesarajah, V., F. M. Schell, D. Gerhold, and G. Stacey. 1987. Cell surface polysaccharides from *Bradyrhizobium japonicum* and a nonnodulating mutant. *J. Bacteriol.* **169**:137–141.
- Rolin, D. B., P. E. Pfeffer, S. F. Osman, B. S. Szwergold, F. Kappler, and A. J. Benesi. 1992. Structural studies of a phosphocholine substituted β -(1,3); (1,6) macrocyclic glucan from *Bradyrhizobium japonicum* USDA 110. *Biochim. Biophys. Acta* **1116**:215–225.
- Schirmer, E. C., J. R. Glover, M. A. Singer, and S. Lindquist. 1996. HSP100/Clp proteins: a common mechanism explains diverse functions. *Trends Biochem. Sci.* **21**:289–296.
- Torres-Cabassa, A. S., and S. Gottesman. 1987. Capsule synthesis in *Escherichia coli* K-12 is regulated by proteolysis. *J. Bacteriol.* **169**:981–989.
- Wawrzynow, A., B. Bannecki, and M. Zylicz. 1996. The Clp ATPases define a novel class of molecular chaperones. *Mol. Microbiol.* **21**:895–899.
- Zevenhuizen, L. P. T. M., and A. R. W. Van Neerven. 1983. (1 $\rightarrow$ 2)- β -D-glucan and acidic oligosaccharides produced by *Rhizobium meliloti*. *Carbohydr. Res.* **118**:127–134.